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Characterization of a multifunctional biocontrol strain, *Bacillus subtilis* CH6, and its antagonistic mechanism against *Pyricularia oryzae* causing rice blast disease

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ABSTRACT: Rice blast disease, caused by *Pyricularia oryzae*, is economically significant and reduces rice productivity. Chemical control methods have proven successful and are widely adopted for managing rice blast disease, but this practice is detrimental to the ecosystem and living creatures. Hence, research on environmentally friendly alternatives such as employing microorganisms to manage plant diseases has attracted significant attention. This study investigated the underlying mechanisms of *Bacillus subtilis* CH6 antagonistic activity against *P. oryzae*. The antagonistic activity of this strain was demonstrated by the dual culture assay, in which fungal growth was inhibited by 86.23%. The bacteria exhibited capacities to produce cell wall degradation enzymes, antifungal secondary metabolites, and volatile organic compounds (VOCs). Furthermore, strain CH6 displayed characteristics of plant growth-promoting bacteria including the ability to produce siderophores, indole-3-acetic acid (IAA) (3.27 ± 0.04 µg/ml), nitrogen fixation activity, as well as phosphate solubilizing activity. The evaluation of bacterial culture extracts indicated the presence of bioactive metabolites which exerted inhibitory activity against both fungal spore germination and mycelial growth, with MIC_{90} values of 0.375 and 60 mg/ml, respectively. Chemical analysis using ultra-high performance liquid chromatography-high resolution tandem mass spectrometry (UHPLC-MS/MS) confirmed the production of fengycins and surfactins which could be responsible for the antagonistic activity of *B. subtilis* CH6 against *P. oryzae*. This investigation provides new insights into the versatility of *B. subtilis* CH6 and its capability of controlling *P. oryzae* as a promising candidate for the development of dual-functioned biocontrol products.

KEYWORDS: Antagonistic activity; *Bacillus subtilis*; biocontrol; Secondary metabolite production, blast disease

INTRODUCTION

Blast disease, caused by *Pyricularia oryzae* (Cook) Sacc. (teleomorph: *Magnaporthe oryzae*), is one of the most devastating fungal infections affecting rice (*Oryza sativa* L.) [1] and is considered the greatest global threat to rice production [2]. Blast disease is common in many rice-growing locations around the world, particularly in tropical and subtropical locations where climatic circumstances favor disease development and dissemination [3]. Fungal infection can appear at all growth stages of rice, and disease symptoms can be observed in any aerial parts, reducing rice yield by 10–30% in general and up to 80% in susceptible rice cultivars such as aromatic rice Khao Dawk Mali 105 (KDML 105) which is planted on half of Thailand's total rice cultivation area [4].

Numerous management practices including chemical and biological control, disease prediction, and traditional breeding methods have been employed to address the damages caused by blast disease [5]. Among these practices, chemical control is recognized as the principal method for managing rice diseases because of its effectiveness, ease of use and affordability [6]. However, the excessive application of synthetic fungicides has led to increasing environmental contamination, potential health risks for humans and animals, and the emergence of fungicide-resistant pathogens, which is not conducive to the sustainable development of rice agriculture [7]. Consequently, biological control by beneficial microorganisms has been suggested as a suitable alternative to the conventional chemical approaches for disease management. The biological control approach is safe and environmentally friendly and also increases the options available to organic rice farmers who cannot use pesticides during cultivation [8].

Bacillus spp. are spore-forming bacteria that are adaptable to a broad range of environmental conditions [9]. They are considered to be a promising group of beneficial microorganisms used as a biological control for plant diseases due to their ability to

produce a wide range of secondary metabolites such as lipopeptides (iturin A, fengycins, and surfactins), polypeptides, macrolactones, fatty acids, polyketides, bacteriocins (lantibiotics, pediocins or thuricins), and isocoumarins [10]. These bacteria have a broad spectrum of antimicrobial activity [11], trigger host defense responses, produce cell wall degradation enzymes (chitin, glucans, mannans, glycoproteins), and possess plant growth-promoting activities. Moreover, *Bacillus* produces gaseous metabolites designated as VOCs which either destroy plant pathogens directly by permeating through their cell wall, or indirectly by inducing plant systemic resistance [12, 13]. Among the *Bacillus* species, *B. subtilis* is one of the most interesting. *B subtilis* strain was reported to possess a large number of gene clusters involved in the biosynthesis of secondary metabolites that have promising activities against many plant pathogens [14]. Cyclic lipopeptides in the surfactin, iturin, and fengycin families exhibit antimicrobial activity against plant pathogens by disrupting their cell membranes. These lipopeptides inhibit the growth of *P. oryzae*, *Colletotrichum gloeosporioides*, and *Bipolaris oryzae* [15]. Considering the vast potential of strain CH6 for agricultural applications, a comprehensive characterization of antifungal compounds synthesized by antagonistic strains is necessary to determine the possible risk associated with their usage.

This paper described *B. subtilis* CH6, isolated from soil in a sugar cane plantation [16]. This strain exhibited antagonistic activity against different plant pathogenic microorganisms such as *Ralstonia solanacearum* [17, 18] and *Pectobacterium carotovorum* subsp. *carotovorum* [16]. This study evaluated the antagonistic activity of strain CH6 against the airborne fungus *P. oryzae* which causes rice blast disease. To investigate its mechanism of action, the main antifungal metabolites produced by strain CH6 were characterized using UHPLC-MS/MS; and its plant growth-promoting capacities were also evaluated. The study findings provided a mechanistic insight into the plant pathogen suppressing and plant growth-promoting activities of *B. subtilis* CH6, which would be essential in future development of this biocontrol agent for the control of blast disease in rice.

MATERIAL AND METHODS

Plant material and microorganisms

The aromatic rice cultivar susceptible to *P. oryzae*, Khao Dawk Mali 105 was used in this study. The fungal pathogen, *P. oryzae* NST040101, isolated from leaves of infected rice, was obtained from Molecular Plant Pathogenic Fungi Laboratory, Department of Plant Pathology, Faculty of Agriculture at Kamphaeng Saen, Kasetsart University Kamphaeng Saen Campus, Nakhon Pathom, Thailand. The antagonistic bacterium, *B. subtilis* CH6 was generously provided by Professor Dr. Niphone Thaveechai, Department of Plant Pathology, Faculty of Agriculture, Kasetsart University, Thailand.

In vitro antagonistic assay

The antagonistic activity against pathogenic fungi was evaluated using a dual culture technique. *P. oryzae* NST04010 was cultivated on potato dextrose agar (PDA) (HiMedia, Mumbai, India) for 14 days. An agar plug containing fungal hyphae was placed at the center of a PDA plate. Then *B. subtilis* CH6 from a colony grown on nutrient agar (NA) (HiMedia) was streaked perpendicularly at 3 cm away from the fungal agar plug (Fig. S1). A control plate was inoculated only with the fungal agar plug. The dual culture plates were incubated at room temperature (30–33 °C) for 14 days. All experiments were conducted in triplicate. Antagonistic activity was determined from the inhibition of growth using the following equation, where D_c represents the diameter of the fungal colony on the control plate, and D_E represents the diameter of the fungal colony culture with *B. subtilis* CH6.

$$\text{Inhibition of fungal growth}\,(\%) = \frac{D_c - D_E}{D_c} \times 100 \quad (1)$$

Antagonistic mechanisms of *B. subtilis* CH6

Production of cell wall degradation enzymes

The contribution of cell wall degradation enzymes, including protease and chitinase, to the antagonistic activity of *B. subtilis* CH6 against *P. oryzae* NST040101 was examined. An agar piece from the inhibition zone between *B. subtilis* CH6 and *P. oryzae* NST040101 from the dual culture experiments described above was excised using a sterile straw (6 mm in diameter) and then placed onto skimmed milk agar (0.5% tryptone, 0.25% yeast extract, 0.1% glucose, 2.8% skimmed milk powder, and 2.0% agar) and chitin agar (0.4% chitin (colloidal), 0.03% KH_2PO_4, 0.07% K_2HPO_4, 0.05% $MgSO_4 \cdot 7H_2O$, 0.001% $MgSO_4 \cdot 7H_2O$, 0.0001% $ZnSO_4 \cdot 7H_2O$, 0.0001% $MnCl_2 \cdot 4H_2O$, and 2.0% agar) to evaluate the production of protease and chitinase, respectively. Assay plates were incubated at room temperature for 24 h before observation of the clear zone indicating protease and chitinase activities around antagonistic agar plugs.

Determination of antifungal activity

Antifungal activity produced by *B. subtilis* CH6 was examined using the agar well diffusion assay with slight modifications. Briefly, one loopful of strain CH6 was inoculated in a test tube containing 5 ml of potato dextrose broth (PDB) (HiMedia), and cultivation was performed at room temperature using orbital shaker (N-Biotek, Bucheon, Korea) at 200 rpm for 72 h. The bacterial culture supernatant was centrifuged at 12,000 rpm for 5 min (Kubota 3700, Tokyo, Japan) to

remove cell debris and, then, sterilized using a 0.2 µm Puradisc™ syringe filter (Whatman, Fontenay Sous Bois, France). NA plates containing 20 ml medium were prepared using a 10 mm diameter cork borer to create a well at 1.5 cm from the center of the agar plate. The agar well was filled with 100 µl of sterilized bacterial culture supernatant. Then, a *P. oryzae* NST040101 agar plug was placed 3 cm away from the well. Instead of sterilized culture supernatant, PDB was used as a negative control. All experiments were conducted in triplicate and incubated at room temperature for 14 days. The observed inhibition zone was measured from the edge of the zone to the edge of the well. Inhibition of fungal growth was calculated using Eq. (1).

Volatile organic compounds (VOCs) production

Examination of VOCs against mycelial production was performed using closed Petri dishes according to the method of Liu et al [19]. In brief, *P. oryzae* NST040101 was incubated on a PDA plate at room temperature for 14 days. Then, a mycelial agar plug (0.6 cm diameter) from the edge of the *P. oryzae* NST040101 colony was transferred onto an 85 mm diameter PDA plate. Another PDA plate was spread with 100 µl of *B. subtilis* CH6 cell suspension (approximately 10^8 CFU/ml) and placed upside down on top of the plate containing the fungal agar plug. The two inoculated plates were then sealed with parafilm to prevent loss of VOCs and incubated at room temperature for 14 days (Fig. S2). The control plate was performed using an uninoculated agar plate in place of *B. subtilis* CH6 culture. The diameter of the *P. oryzae* NST040101 colony on each plate was measured. The fungal inhibition of mycelial growth by the VOCs produced by *B. subtilis* CH6 was calculated using the following Eq. (2):

$$\text{Fungal inhibition by VOCs}\,(\%) = \frac{D_{\text{ck}} - D_{\text{VOCs}}}{D_{\text{ck}}} \times 100 \quad (2)$$

where D_{ck} and D_{VOCs} are the average diameter of *P. oryzae* NST040101 colonies grown on PDA without or with the VOC treatment, respectively.

Plant growth promotion assay

Siderophore production

Qualitative siderophore production was analyzed using chrome azurol S (CAS) agar of Louden et al [20]. A colony of *B. subtilis* CH6 was spotted on CAS agar and incubated at room temperature for 14 days. To assess siderophore production, the formation of yellow to orange halos around the bacterial colonies was observed.

Indole-3-acetic (IAA) production

IAA production was determined according to the method of Gang et al [21]. *B. subtilis* CH6 inoculum was prepared from a 24 h bacterial culture in 20 ml of NB in a 100 ml Erlenmeyer flask. After centrifugation at 8,000 rpm for 15 min, a cell pellet was collected and washed twice with sterile 0.85% (w/v) NaCl. The bacteria cells were resuspended in sterilized 0.85% (w/v) NaCl, and the optical density (OD) at 600 nm was adjusted to 1.0 (Genesys 30, Thermo Scientific, Waltham, USA), equivalent to 10^8 CFU/ml. To evaluate IAA production, 0.5 ml of bacterial inoculum was added into 4.5 ml of NB with and without supplementation of 0.5% (w/v) DL-tryptophan (Sigma-Aldrich, Taufkirchen Germany). The assay was performed in triplicate, and bacterial cultures were incubated in the dark with shaking at 200 rpm at 37 °C for 3 days. After the incubation, the bacterial suspension was centrifuged at 12,000 rpm for 5 min, and the supernatant was collected. Salkowski reagent (1 ml of 0.5 M ferrous chloride in 50 ml of 35% perchloric acid), was added to the supernatant at a ratio of 1:1 (v/v). The mixture was then left in the dark for 30 min at room temperature. The development of a pink color indicated the production of IAA. IAA production was quantified by measuring the absorbance at 535 nm using a spectrophotometer, a standard curve was generated with various concentrations of standard IAA (10–100 µg/ml).

Phosphate solubilization

The ability of bacteria to solubilize phosphate was examined using Pikovskaya's agar (HiMedia). *B. subtilis* CH6 colony was spotted on the test medium and incubated at room temperature for 14 days. The halo zone around the bacterial colony indicated phosphate solubilization.

Potassium solubilization

The potassium solubilizing activity of *B. subtilis* CH6 was examined using Aleksandrow agar (HiMedia). *B. subtilis* CH6 colony was spotted on the test medium and incubated at room temperature for 14 days to observe halo zone formation.

Nitrogen fixation

Nitrogen fixing capability was examined on Okon's nitrogen-free medium (Okon). The pure *B. subtilis* CH6 colony was spotted on the Okon and incubated at room temperature for 72 h. The bacteria capable of nitrogen fixation alkalized the medium which then turned blue due to the generation of ammonia.

Investigation of bioactive metabolites

Preparation of B. subtilis CH6 culture extracts

To investigate the secondary metabolites responsible for the antifungal activity of *B. subtilis* CH6, culture extracts were prepared from the bacterial cultures. *B. subtilis* CH6 was cultivated in 200 ml PDB in a 500 ml Erlenmeyer flask and incubated at room temperature with 200 rpm shaking for 72 h. A portion of

the bacterial culture was extracted with ethyl acetate. In brief, an equal volume of ethyl acetate (analytical grade, Kemaus, Sydney, Australia) was added to the bacterial culture and mixed by shaking on a rotary shaker at 200 rpm for 30 min. The solvent phase was collected and evaporated using a rotary evaporator (Heidolph, Schwabach, Germany).

For the detection of antimicrobial lipopeptides, cell-free culture filtrate was extracted by acid precipitation followed by methanol extraction. In brief, the culture filtrate was adjusted to pH 2.0 by the addition of 6 M hydrochloric acid (analytical grade and KemAus™, New South Wales, Australia), then kept at 4 °C overnight and centrifuged at 3,500 rpm for 10 min using a Sorvall Legend XT Centrifuge (Thermo Fisher Scientific) to collect the precipitate. The precipitate was evaporated, redissolved with distilled water and neutralized to pH 7.0 with 2 M $NaHCO_3$ (Sigma-Aldrich, Missouri, USA), followed by extraction with methanol (RCI Labscan, Bangkok, Thailand). The methanol soluble fraction was dried by evaporation and stored for subsequent bioassays and chemical analysis.

Preparation of P. oryzae NST040101 spores

The conidia of *P. oryzae* NST040101 were prepared according to Chen et al [22] with some modifications. In brief, a 4-day-old colony of *P. oryzae* NST040101 was cut into pieces and transferred to a 250 ml Erlenmeyer flask containing 100 ml of prune broth (clarified prune juice 1.5%, soluble starch 0.25%, yeast extract 0.05%, dH_2O 100 ml, pH 6.5). The fungal culture was incubated in the dark at 28 °C with shaking at 100 rpm for 4 days in an incubator shaker Innova43™ (New Brunswick Scientific Co, New Jersey, UK). The fungal culture was then homogenized at low speed for 15 s in a sterile blender (BRAUN Miniprimer3, Waiblingen, Germany) to obtain liquid spawn. Then, 1 ml of the liquid spawn was dispersed evenly on a 9-cm diameter rice bran agar (3% rice bran powder, 0.2% yeast extract, and 2% agar) plate and air dried to remove excess water in a laminar flow cabinet. The plates were incubated at 28 °C with a full spectrum light emitting diode (LED) slim set T5 plant growth light (Lampton, Bangkok, Thailand) set at a 12 h photoperiod for 4 days. Conidia were harvested by flooding the plates with 10 ml of sterile water containing 0.025% (w/v) Tween 80 and scraping with a glass slide. Spore concentration was determined using a hemacytometer (Hausser Scientific, Horsham, PA, USA).

Determination of spore germination inhibition

Extracts from *B. subtilis* CH6 cultures were tested against *P. oryzae* NST040101 spores to investigate the mechanism by which the bioactive metabolite suppressed the pathogen. The spore germination inhibition assay was performed using the method described by Chutrakul [23]. *B. subtilis* CH6 culture extract samples at six concentrations were prepared by 2-fold serial dilution in 5% (v/v) dimethyl sulfoxide (DMSO). Fungal spores were harvested from the fungal colony and resuspended in 20% (v/v) minimal medium (MM) to a concentration of 8×10^4 spores/ml, and 25 µl of spore suspension was dispensed into each well of a 384-well clear flat bottom polystyrene TC-treated microplate (Corning, Glendale, USA). The plate was incubated at 25 °C for 3–3.5 h to allow spore attachment. Then, an equal volume of extract solution in 5% (v/v) DMSO, 5% (v/v) DMSO (negative control) or amphotericin B (Sigma-Aldrich) (positive control) solution in 5% (v/v) DMSO, was added and allowed to incubate for 16–18 h. Germinating spores were examined using an inverted microscope (Olympus IX71, Olympus, Tokyo, Japan) at 100× magnification and subsequently stained by adding 25 µl of 0.067 mg/ml 5(6)-carboxyfluorescein diacetate (CFDA) in 40% glycerol to each well and incubated in the dark for 10 min. The plate was then rinsed with tap water and blotted using a paper towel to remove residual water. Twenty-five microliters of distilled water were added to the wells, and fluorescence intensity was measured using a microplate reader (Synergy H1, BioTek Instruments, Inc., Vermont, USA) for excitation and emission wavelengths at 485 and 535 nm, respectively. The average fluorescence unit (FU) values obtained from triplicate samples were used to calculate the inhibition of spore germination using the following Eq. (3):

$$\text{Inhibition of spore germination (\%)} = \left[1 - \frac{FU_{T\,\text{sample}} - FU_{T0\,\text{sample}}}{FU_{T\,DMSO} - FU_{T0\,DMSO}}\right] \times 100 \quad (3)$$

where $FU_{T\,\text{sample}}$ and $FU_{T0\,\text{sample}}$ represent average FU values of sample wells at the endpoint and the time zero, respectively, and $FU_{T\,DMSO}$ and $FU_{T0\,DMSO}$ represent average FU values of negative control wells at the endpoint and the time zero, respectively. To determine the minimal inhibitory concentration at 90% inhibition (MIC_{90}), each sample was tested in triplicate at six concentrations, prepared by 2-fold serial dilution.

Determination of mycelial growth inhibition

The activity of *B. subtilis* CH6 culture extract against the mycelial growth of *P. oryzae* NST040101 was investigated. Briefly, culture extracts prepared by ethyl acetate extraction were dissolved in 1% (v/v) DMSO and sterilized through a 0.2 µm Puradisc™ syringe filter. Agar plugs containing *P. oryzae* mycelia were placed at the center of the PDA plates supplemented with different concentrations of culture extract, ranging from 0.03 to 60 mg/ml and incubated at 25 °C for 14 days. A PDA plate containing 0.1% (v/v) DMSO was used as a negative control. All experiments were conducted in triplicate. The radius of *P. oryzae* was

recorded and the inhibition ration was calculated using the following Eq. (4):

$$\text{Inhibition ration}(\%) = \frac{\text{A1} - \text{A2}}{\text{A1}} \times 100 \qquad (4)$$

where A1 and A2 represent the radii of *P. oryzae* in the control and the treatment plates, respectively.

Characterization of B. subtilis CH6 antifungal metabolites by UHPLC-MS/MS

B. subtilis CH6 culture extracts were analyzed by UHPLC-MS/MS using a Dionex UltiMate 3000RS UHPLC system (Thermo Scientific) coupled with an Orbitrap Fusion Tribrid mass spectrometer (Thermo Scientific). Samples were separated on an ACQUITY UPLC BEH C18 column (2.1 × 100 mm, 1.7 µm) (Waters, MA, USA) using a mobile phase consisting of water containing 0.02% (v/v) formic acid (A) and acetonitrile containing 0.016% (v/v) formic acid (B) under the following gradient conditions: 0–0.17 min, 5% B; 0.17–8.40 min, 5% B to 100% B; 8.40–10.45 min, 100% B; 10.45–10.50 min, 100% B to 5% B; and 10.50–12.50 min, 5% B. Extract samples were dissolved in LC-MS grade methanol at a concentration of 0.5 mg/ml and injected at a volume of 3 µl. The flow rate of the mobile phase was maintained at 0.4 ml/min, and the column temperature was set at 60 °C.

Mass analysis was performed on a mass spectrometer equipped with an electrospray ionization (ESI) source and operated with a target mass resolution of 120,000 and acquisition range between 120 and 2,000 m/z. Mass spectrometer conditions were set with the ESI voltage at 3,500 V, capillary voltage at 3.5 kV, ion transfer tube temperature at 333 °C, and vaporizer temperature at 317 °C. Mass spectra were acquired in full scan and in the parallel reaction monitoring (PRM) mode, using high energy collisional dissociation (HCD) energy of 30 ± 10% in both positive and negative ionization modes. Mass spectral analysis was performed using a FreeStyle (Thermo Scientific). MS/MS data were annotated by searching against the Global Natural Products Social Molecular Networking (GNPS) public library and compared with previous reports.

Statistical analysis

All experiments were performed in triplicate. Each analysis was carried out using the least significant difference test ($p < 0.05$) following one-way analysis of variance (ANOVA) using SPSS ver. 22.0 software for Windows (SPSS Inc., Chicago, IL, USA). All data were expressed as mean values with standard deviation (± SD).

RESULTS AND DISCUSSION

Biocontrol activity and mechanisms of *B. subtilis* CH6 against *P. oryzae*

The strong antagonistic activity of *B. subtilis* CH6 against *P. oryzae* was shown in a dual culture assay, in which fungal growth was inhibited by 86.23 ± 0% (Fig. 1). The biological control of pathogens can occur through a combination of mechanisms [24] including antibiosis, cell wall degradation enzymes production, VOCs production, and induction of plant defense response [25]. To determine the mechanisms by which *B. subtilis* CH6 suppressed *P. oryzae*, the capacities of this strain to produce various bioactive components such as cell wall degradation enzymes, antifungal metabolites, and VOCs were evaluated.

The role of cell wall degradation enzymes in the antagonistic activity of *B. subtilis* CH6 against *P. oryzae* was investigated. Results revealed that *B. subtilis* CH6 produced protease under the assay conditions (Fig. 2a). The production of protease might contribute to the disruption of the fungal cell wall, with major constituents chitin, glucans, and glycoproteins [26]. However, the chitinase assay showed a negative result, despite prolonged cultivation time (21 days) (data not shown), which suggested the lack of capacity for chitinase production. The absence of chitinase activity during antagonism suggested that this strain might not be able to produce or express the chitinase enzyme.

The production of antifungal substances and VOCs was investigated. The production of antifungal compounds was examined using PDB, as the same medium used during the screening of antagonistic activity. Results showed that strain CH6 produced antifungal substances in the culture broth with inhibition percentage of 32.29 ± 0.20% (Fig. 2c,d). This finding concurred with a previous report of antifungal metabolites produced by *Bacillus* spp. against *A. solani*, *B. cinerea*, and *Magnaporthe oryzae* [27]. Antifungal substances requière evaluation for clarity and safety when used in agriculture.

VOCs have been proven to control plant pathogens, stimulate plant growth, and induce systemic disease resistance [28]. To examine the contribution of VOCs in the antagonistic activity of strain CH6, the bacteria was tested against *P. oryzae* using the closed Petri dish assay method. Results showed that strain CH6 produced VOCs which reduced the growth of *P. oryzae* by 64.82 ± 0.69% (Fig. 2e–g). The role of VOCs in the antagonistic activity of *Bacillus* has been recognized by many researchers. Wu et al [29] showed that two VOCs produced by strain L3 exhibited strong antifungal properties against *Fusarium oxysporum* f. sp. *niveum*, while other VOCs such as acetoin and 2,3-butanediol were found to promote plant growth. However, not all species of *Bacillus* have the capacity to produce

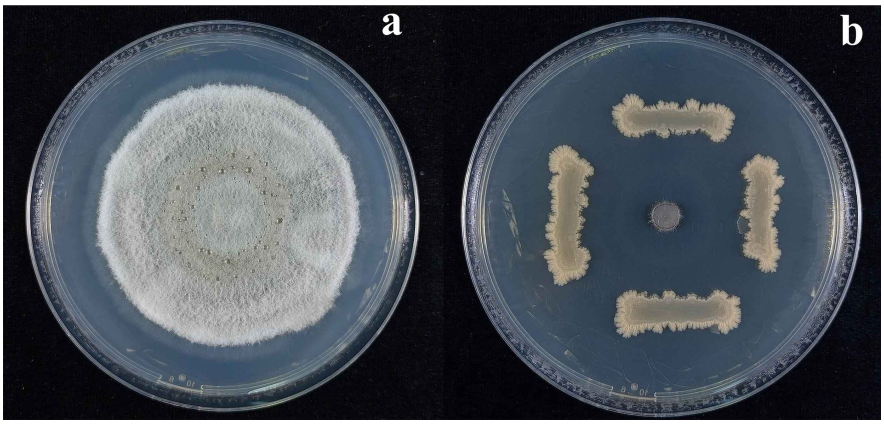

Fig. 1 Antagonism assay on PDA medium: (a), control *P. oryzae* colony; and (b), dual culture of *B. subtilis* CH6 against *P. oryzae* after 14-day cultivation at room temperature (30–33 °C).

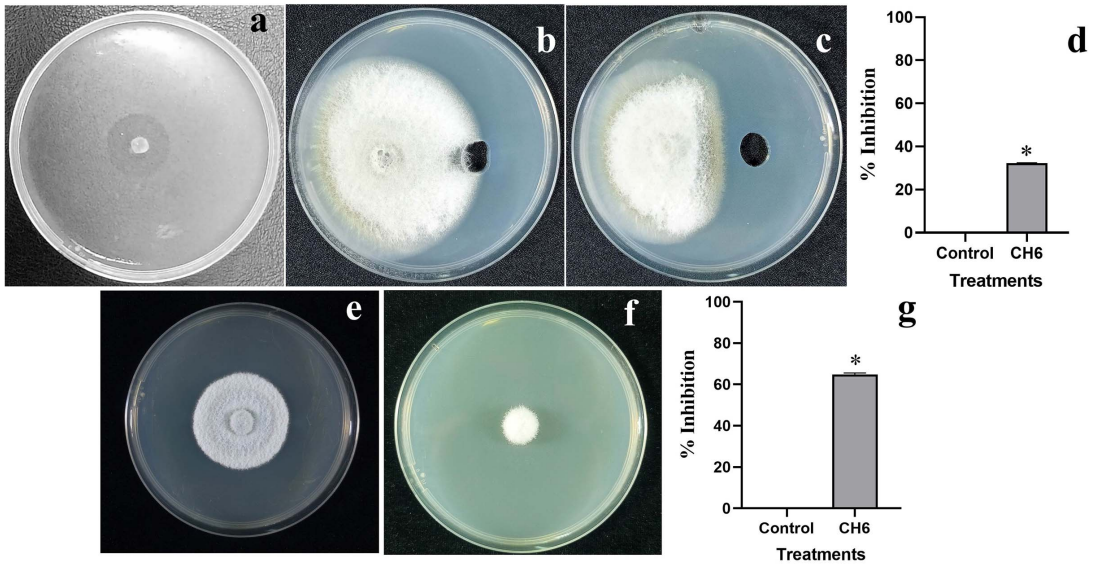

Fig. 2 Mechanisms of *B. subtilis* CH6 antagonistic activity: (a), protease production; (b–d), antifungal production; (b), control *P. oryzae* colony; (c), antagonism assay by well diffusion; (d), percentage of fungal growth inhibition by strain CH6; and (e–g), VOC production; (e), control *P. oryzae* colony; (f), *P. oryzae* colony treated with VOCs; and (g), percentage of inhibition of fungal growth by VOCs.

VOCs [30]. Results showed that the suppression of mycelial growth of *P. oryzae* was mainly due to diffusible antifungal substances such as protease and antifungal secondary metabolites produced by *B. subtilis* CH6.

Plant growth promotion ability of *B. subtilis* CH6

A significant amount of literature has focused on the potential applications of plant-associated bacteria, which can function as agents that promote plant growth through nutrient uptake from the soil, with the production and release of phytohormones serving as indications of soil quality and plant health [31]. *Bacillus* species have been extensively studied for their ability to suppress plant pathogens and enhance plant fitness [32]. Therefore, biocontrol methods employing antagonistic microorganisms associated with plant promotion activities offer an attractive option for the development of alternative agricultural tools to control plant pathogenic microorganisms [33]. The antagonistic bacterium, *B. subtilis* CH6 was investigated for plant growth-promoting capabilities including phytostimulant production and nutrient solubilization.

IAA is a phytohormone of the auxin class that regulates the growth of plant roots through the stimulation of root cell proliferation and elongation [34]. IAA production by strain CH6 was quantitatively determined. The results demonstrated that this strain

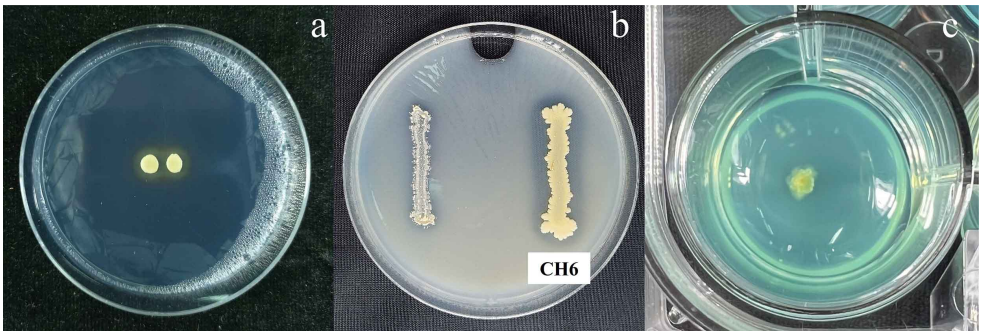

Fig. 3 Plant growth promotion properties of *B. subtilis* CH6 demonstrated by assays for siderophore production on: (a), CAS medium; (b), phosphate solubilization on Pikovskaya's medium; and (c), nitrogen fixation on Okon's nitrogen-free medium. The assays were performed by cultivating *B. subtilis* CH6 on corresponding media at room temperature (30–33 °C) for 14 days for siderophore and phosphate solubilization assays (a and b) and 3 days for nitrogen fixation assay (c).

secreted IAA at a concentration of 3.27 ± 0.04 μg/ml when cultivated in LB medium supplemented with 0.5% (w/v) tryptophan. By contrast, bacterial culture in LB without tryptophan did not show any observable level of IAA production. This result correlated with previous studies, suggesting that the application of low IAA concentrations (about 1 nmol/l to 10 μg/l) promotes plant growth, whereas a higher concentration of IAA has the opposite effect on plants [35].

The function of siderophores synthesized by *Bacillus* species has been well documented as iron-chelating agents that directly facilitate iron mobilization within plants. Siderophores also inhibit the growth of plant pathogens by scavenging iron from the environment, resulting in a deficiency of iron necessary for their growth [28, 36]. Various investigations have studied plant growth-promoting rhizobacteria (PGPR) as a potential tool for plant disease management via the production of plant-beneficial metabolites such as siderophores [37]. Strain CH6 showed a yellow halo zone around the bacterial colony on CAS medium (Fig. 3a), indicating the ability of this strain to secrete siderophores and its potential for use as a microbial agent that increased iron availability for the plant and limited the growth of plant pathogens through iron competition.

Phosphorus and potassium are essential macro elements in plants [38]. Phosphorus is present in the soil as insoluble phosphates which plants cannot absorb. Between 90 and 98% of soil potassium is mineral and mostly inaccessible for plant absorption [39]. Nevertheless, various phosphate- and potassium-solubilizing microbes are known to efficiently make unused phosphate and potassium accessible to plants by releasing organic acids and solubilizing enzymes [40]. This study investigated the capability of *B. subtilis* CH6 to solubilize these essential elements using test media containing inorganic phosphate and potassium. Results showed that strain CH6 solubilized phosphate, as indicated by a halo zone around the bacterial colony grown on Pikovskaya's agar (Fig. 3b). However, no hydrolytic halo was formed around strain CH6 colonies on Aleksandrow agar (data not shown), indicating the absence of potassium solubilizing activity.

Nitrogen (N) is essential for plant growth [41]. The nitrogen fixing capacity of strain CH6 was examined in Okon medium. The result showed that strain CH6 could convert molecular dinitrogen (N_2) to ammonia (NH_3), which indicated the transformation of a medium from yellow to blue (Fig. 3c). Various strains of PGPR in *Bacillus* sp., particularly *B. subtilis*, were identified as asymbiotic nitrogen-fixing bacteria with the capacity to reduce chemical fertilizer-N use and increase plant growth and yield [42].

Our results indicated that the antagonistic bacteria, *B. subtilis* CH6, showed potential for use as a multifunctional biocontrol agent, providing plant protection and serving as a biofertilizer.

Investigation of antifungal secondary metabolites produced by *B. subtilis* CH6

Results from the agar well diffusion assay suggested the role of soluble substances in the antifungal activity of *B. subtilis* CH6 against *P. oryzae*, and the production of small molecule metabolites responsible for this activity was investigated. Bacterial culture in PDB was extracted by ethyl acetate to recover a wide range of polar and non-polar compounds, and subjected to acid precipitation followed by methanol extraction to enrich lipopeptides commonly produced by *Bacillus* species [43].

To determine the mechanism of *P. oryzae* inhibition by the bioactive metabolites from *B. subtilis* CH6, the extract samples were examined for activities against fungal spore germination as well as mycelial growth. The spore germination inhibition assay showed positive results for the extracts under ethyl acetate extraction, as well as for those obtained by acid precipitation and methanol extraction (Fig. S3). The MIC_{90} values of the ethyl acetate extract and the acid

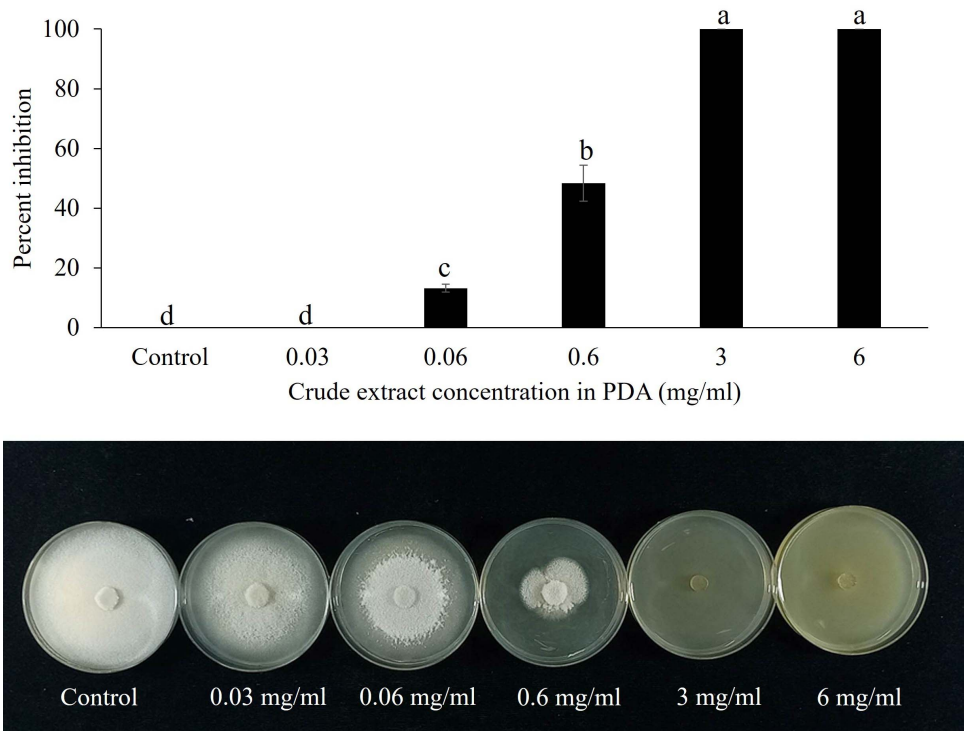

Fig. 4 Inhibitory activity of *B. subtilis* CH6 crude extract against *P. oryzae*. The fungus was grown on PDA plates supplemented with different concentrations of ethyl acetate extract. The bar graph shows the average inhibition percentage of fungal growth at different concentrations of extract. Error bars indicate the standard deviation calculated from triplicated data points. Different letters above the bars indicate significant differences ($p < 0.05$).

precipitate/methanol extract against *P. oryzae* spores were 0.375 and 1.25 mg/ml, respectively, indicating a lower proportion of antifungal metabolites in the latter extract. Hence, only the ethyl acetate extract was evaluated for mycelial inhibitory activity. The inhibitory activity of the extract was evident at a concentration of 0.06 mg/ml, with complete inhibition of fungal growth observed at 3 mg/ml (Fig. 4). These findings indicated that the antifungal substances produced by *B. subtilis* CH6 were effective against both spores and mycelium of *P. oryzae*, corresponding with a previous report on the antifungal activity of *B. velezensis* HY19 against *Botrytis cinerea* [44].

Bacillus spp. are widely recognized for their ability to produce bioactive secondary metabolites. In particular, *B. subtilis* has been demonstrated to produce complex cyclic lipopeptides including surfactins, iturins, and fengycins and other peptides such as bacilysin and macrolactin. Therefore, these metabolites were selected as target molecules UHPLC-MS/MS analysis. Based on the molecular mass and MS fragmentation, 17 lipopeptides including 6 isoforms of fengycin A and 6 isoforms of fengycin B with variable numbers of carbon atoms and branching in their β-hydroxy fatty acid moieties, and 5 isoforms of surfactin with C_{12}—C_{16} β-hydroxy fatty acids identified in both the ethyl acetate and acid precipitate/methanol extracts (Table S1). These series of fengycins and surfactins were detected in both types of extracts from replicates of cultures, suggesting the contribution of these metabolites in the antifungal activity of *B. subtilis* CH6. The failure to detect macrolactin, bacillaene, bacilysin, and mersacidin indicated that the biosynthetic genes involved in the production of these compounds might not be expressed, or might be expressed at low levels under the experimental conditions. These findings correlated with previous research, which indicated that *B. subtilis* could synthesize a wide range of antimicrobial compounds including macrolactin, bacillaene, difficidin, surfactin, bacillomycin-D, fengycin, and the siderophore bacillibactin [14].

CONCLUSION

The application of biocontrol strategies in agriculture can reduce the use of chemical fungicides, improve agro-ecosystems, and maintain a natural balance. However, the safety of biocontrol agents is an essential factor that must not be disregarded. This study highlighted the multifunctional capacities of *B. subtilis* CH6 to produce antifungal agents against blast disease in rice and promote plant growth through the production of phytohormones, IAA, siderophores, nitro-

gen fixation, and solubilizing phosphorus. Fengycins and surfactins were detected as antifungal metabolites involved in the antagonistic activity against *P. oryzae*. This study paves ways for the commercial development of new biological control agents based on *B. subtilis* CH6. To fully explore the potential benefits of this *Bacillus* strain, additional investigations are necessary to examine its effects on plant defense mechanisms and the variations in microbial communities that occur during strain CH6 application.

Appendix A. Supplementary data

Supplementary data associated with this article can be found at https://dx.doi.org/10.2306/scienceasia1513-1874.2024.096.

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Appendix A. Supplementary data

Table S1 Lipopeptides identified from culture extracts of *B. subtilis* CH6 by UHPLC-MS/MS.

No.	Compound	t_R (min)	Positive				Negative		Ref.
			Observed [M+H]+ m/z	mass error (ppm)	Observed [M+2H]2+ m/z	mass error (ppm)	Observed [M−H]− m/z	mass error (ppm)	
Ethyl acetate extract									
1.	Fengycin A C15	5.87	1449.7883	1.66	725.3991	3.31	1447.7690	–	Jumpathong,
2.	Fengycin A C16	5.99	1463.8040	0.00	732.4067	2.05	1461.7859	–	et al, 2022
3.	Fengycin A C17	6.04	1477.8215	2.50	739.4149	3.38	1475.8007	–	
4.	Fengycin A C18	6.15	1491.8375	1.41	746.4228	2.41	1489.8175	–	
5.	Fengycin A C15:1	6.34	1447.8116	2.42	724.4094	2.62	1445.7935	–	
6.	Fengycin A C16:1	6.35	1461.8262	1.30	731.4174	2.46	1459.8062	–	
7.	Fengycin B C15	6.04	1477.8215	3.45	739.4149	4.06	1475.8007	–	
8.	Fengycin B C16	6.15	1491.8367	1.61	746.4227	2.95	1489.8175	–	
9.	Fengycin B C17	6.30	1505.8522	1.53	753.4303	2.92	1503.8319	–	
10.	Fengycin B C18	6.56	–	–	760.4375	2.37	–	–	
11.	Fengycin B C14:1	6.35	1461.8262	1.37	731.4174	1.91	1459.8062	–	
12.	Fengycin B C15:1	6.48	1475.8417	5.29	738.4250	3.25	1473.8218	–	
13.	Surfactin C12	8.24	994.6453	1.31	–	–	992.6262	–	Shahid,
14.	Surfactin C13	8.41	1008.6611	0.10	–	–	1006.6415	–	et al, 2021
15.	Surfactin C14	8.73	1022.6773	1.27	–	–	1020.6564	–	
16.	Surfactin C15	8.89	1036.6916	1.54	–	–	1034.6725	–	
17.	Surfactin C16	9.15	1050.7078	–0.19	–	–	1048.6875	–	
Acid precipitate/methanol extract									
1.	Fengycin A C15	6.45	1449.7909	3.45	725.3985	2.48	1447.7710	–	Jumpathong,
2.	Fengycin A C16	6.56–6.63	1463.8045	0.34	732.4065	1.77	1461.7874	–	et al, 2022
3.	Fengycin A C17	6.69–6.84	1477.8221	2.91	739.4149	3.38	1475.8037	–	
4.	Fengycin A C18	6.70–6.84	1491.8377	1.07	746.4224	2.01	1489.8185	–	
5.	Fengycin A C15:1	6.96	1447.8097	1.11	724.4091	2.21	1445.7917	–	
6.	Fengycin A C16:1	7.01-7.19	1461.8243	0.00	731.4178	3.01	1459.8073	–	
7.	Fengycin B C15	6.59–6.84	1477.8214	3.38	739.4146	3.65	1475.8027	–	
8.	Fengycin B C16	6.70–6.84	1491.8367	1.61	746.4224	2.55	1489.8185	–	
9.	Fengycin B C17	6.13–7.03	–	–	753.4299	2.39	–	–	
10.	Fengycin B C18	6.28–7.43	–	–	760.4373	2.10	–	–	
11.	Fengycin B C14:1	7.01–7.19	1461.8243	0.07	731.4178	2.46	1459.8073	–	
12.	Fengycin B C15:1	7.10–7.29	–	–	738.4253	3.66	–	–	
13.	Surfactin C12	7.76	994.6447	0.70	–	–	992.6284	–	Shahid,
14.	Surfactin C13	7.97	1008.6609	–0.10	–	–	1006.6432	–	et al, 2021
15.	Surfactin C14	8.30	1022.6764	0.39	–	–	1020.5870	–	
16.	Surfactin C15	8.49	1036.6918	1.74	–	–	1034.6737	–	

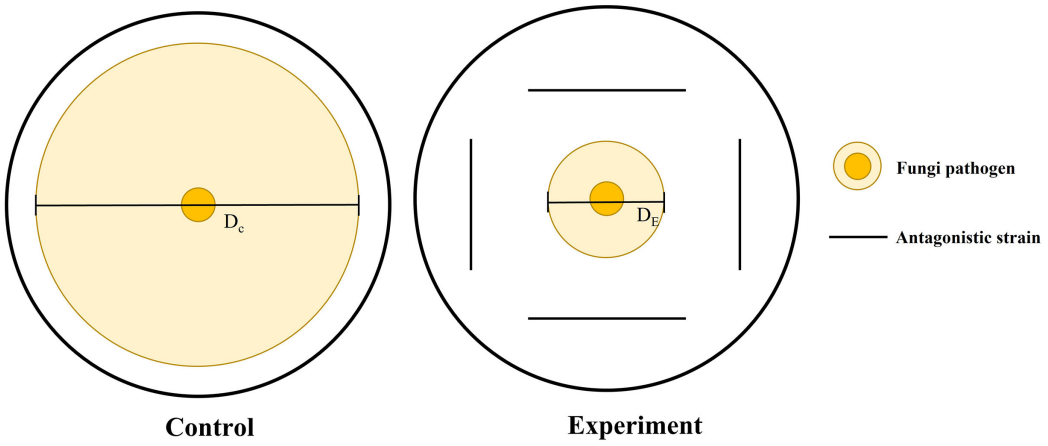

Fig. S1 Dual culture technique for the evaluation *B. subtilis* CH6 antagonistic activity against *P. oryzae* NST040101. Picture was created with BioRender.com.

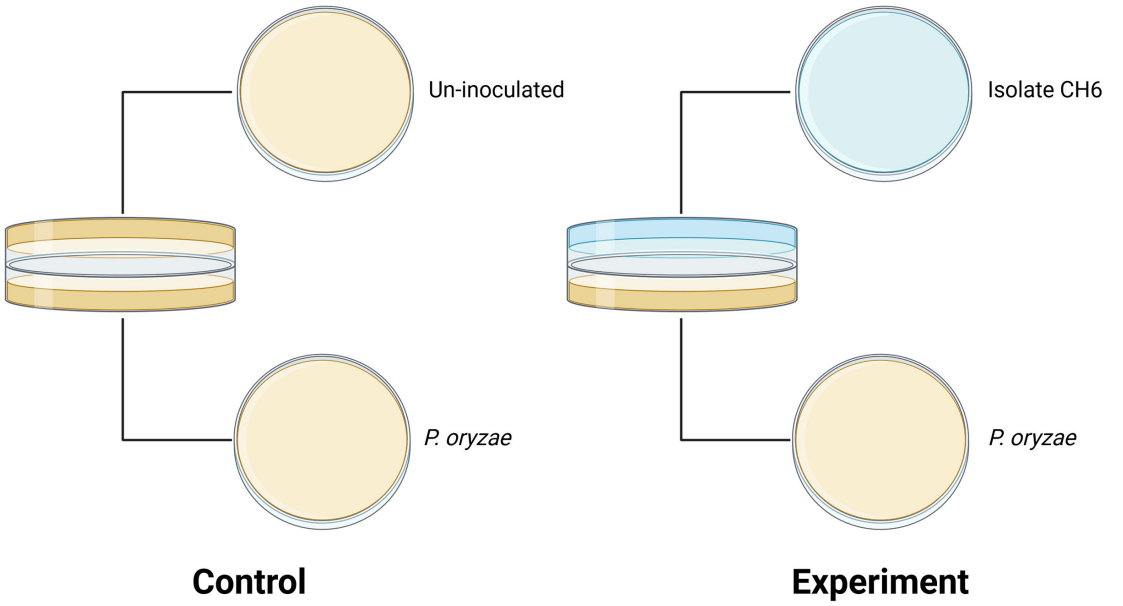

Fig. S2 Schematic depiction of closed Petri dish method for VOC detection. Picture was created with BioRender.com.

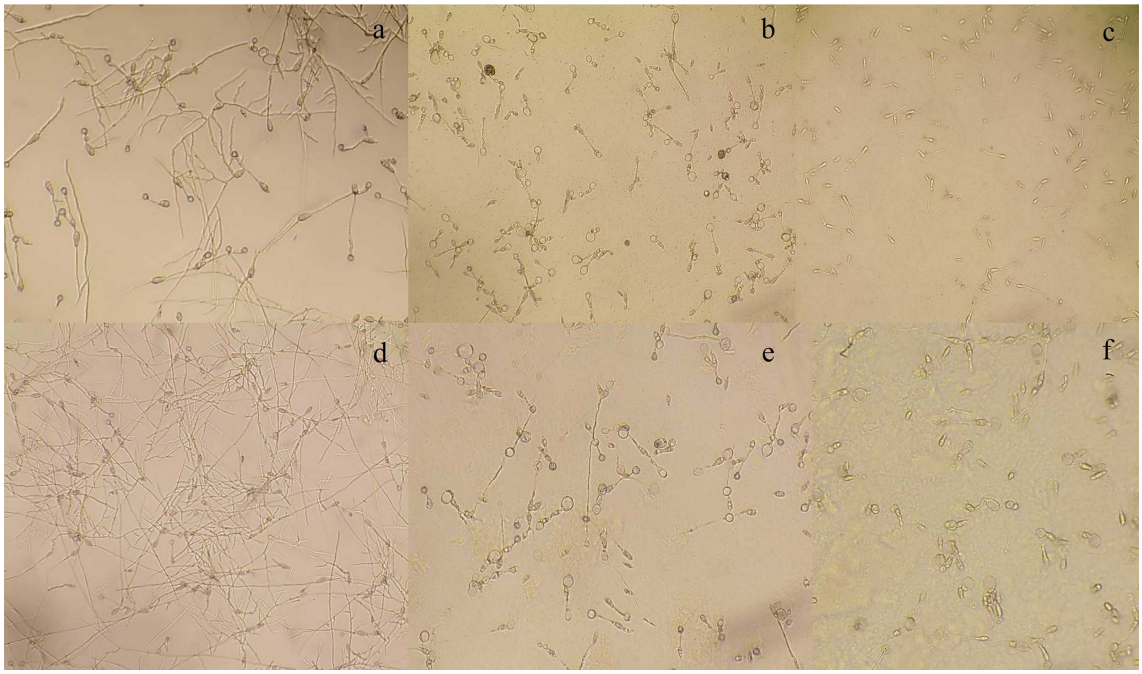

Fig. S3 Effects of *B. subtilis* CH6 culture extracts on the development of *P. oryzae* spores. Images taken from inverted microscope (100× magnification) display the development of *P. oryzae* germlings after 16–18 h exposure to 2.5% (v/v) DMSO (control) (a), 0.19 mg/ml ethyl acetate extract (b), 0.38 mg/ml ethyl acetate extract (c), 2.5% (v/v) methanol (control) (d), 1.25 mg/ml acid precipitate/methanol extract (e), and 2.50 mg/ml acid precipitate/methanol extract (f).